



# N<sup>pro</sup> of classical swine fever virus is an antagonist of double-stranded RNA-mediated apoptosis and IFN- $\alpha$ / $\beta$ induction

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## Abstract

Classical swine fever virus (CSFV) protects cells from double-stranded (ds) RNA-mediated apoptosis and IFN- $\alpha$ / $\beta$  induction. This phenotype is lost when CSFV lacks N<sup>pro</sup> ( $\Delta$ N<sup>pro</sup> CSFV). In the present study, we demonstrate that N<sup>pro</sup> counteracts dsRNA-mediated apoptosis and IFN- $\alpha$ / $\beta$  induction independently of other CSFV elements. For this purpose, we generated porcine SK-6 and PK-15 cell lines constitutively expressing N<sup>pro</sup> fused to the enhanced green fluorescent protein (EGFP). The survival of the SK6-EGFP-N<sup>pro</sup> cell line after polyinosinic polycytidylic acid [poly(IC)] treatment was comparable to that of CSFV-infected SK-6 cells and was significantly higher than the survival of the parent cell line. In PK-15 cells, the presence of EGFP-N<sup>pro</sup> prevented the  $\Delta$ N<sup>pro</sup> CSFV- and poly(IC)-mediated IFN- $\alpha$ / $\beta$  production. Importantly, N<sup>pro</sup> also inhibited IFN- $\alpha$  and IFN- $\beta$  promoter-driven luciferase expression in human cells and blocked IFN- $\alpha$ / $\beta$  induction mediated by Newcastle disease virus. This establishes a novel function for N<sup>pro</sup> in counteraction of the IFN- $\alpha$ / $\beta$  induction pathway. © 2005 Elsevier Inc. All rights reserved.

**Keywords:** Pestivirus; Classical swine fever virus; CSFV; N<sup>pro</sup>; Double-stranded RNA; Interferon- $\alpha$ / $\beta$

## Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever, a severe hemorrhagic disease of pigs. CSFV together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV) belongs to the genus *Pestivirus* within the Flaviviridae family. The other members of the Flaviviridae are the genera *Flavivirus* and *Hepacivirus* that represent important human pathogens (vanRegenmortel et al., 2000). Pestiviruses carry an RNA

genome of positive polarity that possesses a 5' untranslated region (5'UTR), a single open reading frame (ORF) and a 3'UTR. The 5'UTR functions as an internal ribosomal entry site (IRES) for cap-independent translation initiation of the large ORF that is co- and posttranslationally processed into twelve mature proteins by viral and host cell proteases (for review, see Meyers and Thiel, 1996).

The first protein encoded by the ORF is the nonstructural protein N<sup>pro</sup>. The only established function of N<sup>pro</sup> is its autoproteolytic activity that allows cleavage from the downstream nucleocapsid protein C (Rumenapf et al., 1998; Stark et al., 1993; Wiskerchen et al., 1991). Interestingly, N<sup>pro</sup> is unique to the pestiviruses within the Flaviviridae family and was found to be dispensable for virus replication in cell culture (Lai et al., 2000; Tratschin et al., 1998). The N<sup>pro</sup> gene of the moderately virulent strain vA187-1 and the highly virulent strain vEy-37 was deleted or replaced with the murine ubiquitin gene that substitutes for N<sup>pro</sup> in the generation of the authentic nucleocapsid protein. These CSFV mutants lacking the N<sup>pro</sup> gene ( $\Delta$ N<sup>pro</sup> CSFV)

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displayed no major alteration of growth characteristics in the porcine kidney cell line SK-6 but were avirulent in pigs (Mayer et al., 2004; Tratschin et al., 1998). Deletion of viral genes or mutations that did not abolish virus replication in cell culture but resulted in attenuation in the immune-competent host led to the discovery of various mechanisms acquired by viruses during evolution to subvert the host defense, in particular the interferon system (for recent reviews, see Basler and Garcia-Sastre, 2002; Goodbourn et al., 2000; Weber et al., 2004). For the pestiviruses, it is now well established that virus replication interferes with cellular antiviral defense (Baigent et al., 2002; Bensaude et al., 2004; Carrasco et al., 2004; La Rocca et al., 2005; Ruggli et al., 2003; Schweizer and Peterhans, 2001). Our recent data with  $\Delta N^{\text{pro}}$  CSFV showed that  $N^{\text{pro}}$  was required by CSFV to prevent double-stranded (ds) RNA-mediated apoptosis in SK-6 cells and IFN- $\alpha/\beta$  induction in macrophages (Ruggli et al., 2003). Furthermore, as opposed to wild-type CSFV,  $\Delta N^{\text{pro}}$  CSFV induced IFN- $\alpha/\beta$  independently of exogenous dsRNA stimulation, suggesting a role for  $N^{\text{pro}}$  in the counteraction of IFN- $\alpha/\beta$  induction. However, the latter data did not exclude the requirement of other CSFV elements in conjunction with  $N^{\text{pro}}$ . Very recently, it was shown that  $N^{\text{pro}}$  of BVDV was partly responsible for blocking interferon regulatory factor (IRF)3-dependent transcription (Horscroft et al., 2005). Furthermore, while this manuscript was under revision,  $N^{\text{pro}}$  was shown to be involved in transcriptional inhibition of IRF3 (La Rocca et al., 2005). In addition, a recent report suggested a role of BVDV  $E^{\text{ns}}$  in the control of IFN- $\alpha/\beta$  induction (Iqbal et al., 2004). In the present study, we demonstrate that  $N^{\text{pro}}$  of CSFV is capable of preventing polyinosinic polycytidylic acid [poly(IC)]- and Newcastle disease virus (NDV)-mediated IFN- $\alpha/\beta$  induction, independently of the CSFV context. These data clearly establish a novel function for  $N^{\text{pro}}$  in interference with the induction of IFN- $\alpha/\beta$ .

## Results

### *Establishment of porcine SK-6 and PK-15 cell lines expressing the enhanced green fluorescent protein (EGFP)- $N^{\text{pro}}$ fusion protein or EGFP alone*

When treated with poly(IC), SK-6 cells undergo apoptosis but do not produce any detectable IFN- $\alpha/\beta$ . In contrast, PK-15 cells tolerate higher poly(IC) concentrations in the cell culture supernatant and respond to poly(IC) transfection with IFN- $\alpha/\beta$  production. Infection with CSFV, as opposed to CSFV lacking the  $N^{\text{pro}}$  gene, prevents these cellular responses, suggesting that  $N^{\text{pro}}$  is an antagonist of cellular antiviral defense (Ruggli et al., 2003). In order to study the function of  $N^{\text{pro}}$  independently of other CSFV elements, we established stable clonal SK-6 and PK-15 porcine kidney cell lines expressing  $N^{\text{pro}}$  in fusion with EGFP under the control of the human

cytomegalovirus (CMV) immediate early promoter. Control cell lines expressing EGFP alone were generated in order to exclude a non-specific effect due to expression of a foreign protein. After repeated end point dilution, we obtained stable SK-6 and PK-15 clones expressing the EGFP- $N^{\text{pro}}$  fusion protein (SK6-EGFP- $N^{\text{pro}}$ , PK15-EGFP- $N^{\text{pro}}$ ) or EGFP alone (SK6-EGFP, PK15-EGFP). Two independent cell clones were selected for each type of stable transformant. As expected, a 46 kDa protein was detected for the EGFP- $N^{\text{pro}}$  expressing cells with both the anti-EGFP (Fig. 1A, lanes 2 and 5) and the rabbit anti- $N^{\text{pro}}$  (Fig. 1B, lanes 2 and 5) antibodies. A 27 kDa protein was detected for the EGFP expressing cell lines with the anti-EGFP monoclonal antibody (mAb) (Fig. 1A, lanes 3 and 6). For the SK-6 and PK-15 clones, the expression level of the EGFP- $N^{\text{pro}}$  fusion protein (46 kDa) was 10, respectively 60 times lower than the EGFP (27 kDa) expression level. Six times less EGFP- $N^{\text{pro}}$  fusion protein was expressed in the PK-15 clones when compared with the SK-6 clones. The result shown in Fig. 1 for one set of stable transformants is representative of both independent clones.

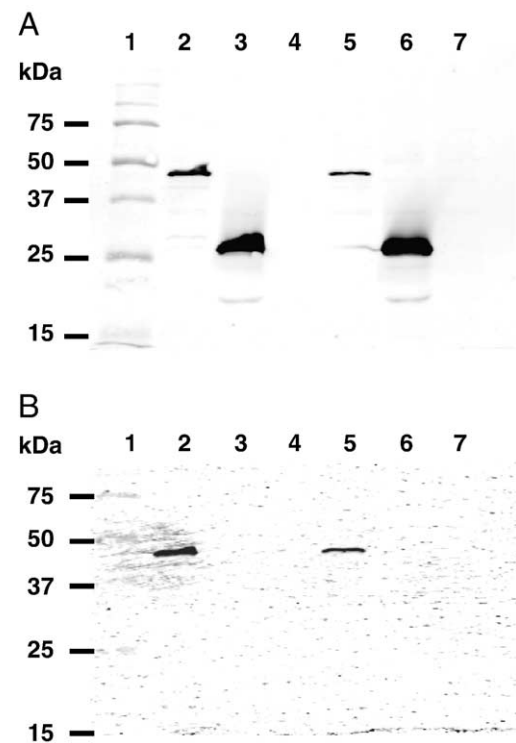


Fig. 1. Western blot analysis of EGFP- $N^{\text{pro}}$  and EGFP expression in stably transformed SK-6 and PK-15 cells. SK-6 and PK-15 cells were transfected with linearized expression plasmid and selected for G418 resistance. Single colonies of stably transformed clones expressing EGFP- $N^{\text{pro}}$  fusion protein (46 kDa) or EGFP alone (27 kDa) were picked and purified by several rounds of end point dilution. Equivalent amounts of protein extract (5  $\mu$ g/lane) from the cloned cell lines were separated by SDS-PAGE and analyzed by Western blotting using mAb JL-8 directed against EGFP (A) and a rabbit antiserum raised against  $N^{\text{pro}}$  (B). Lane 1: molecular weight marker, lane 2: SK6-EGFP- $N^{\text{pro}}$ , lane 3: SK6-EGFP, lane 4: SK-6, lane 5: PK15-EGFP- $N^{\text{pro}}$  #1, lane 6: PK15-EGFP #1, lane 7: PK-15.

*The EGFP-N<sup>pro</sup> fusion does not alter the capacity of CSFV to prevent poly(IC)-mediated apoptosis and IFN- $\alpha/\beta$  induction*

The use of EGFP-N<sup>pro</sup> fusion for the analysis of the IFN- $\alpha/\beta$  antagonistic function of N<sup>pro</sup> was first evaluated in the virus context. For this purpose, the N<sup>pro</sup> gene of the full-length cDNA clone pA187-1 was replaced by the EGFP-N<sup>pro</sup> fusion cassette of the expression plasmid pCMV-EGFP-N<sup>pro</sup>. Because the RNA secondary structure immediately downstream of the start codon is critical for translation initiation of pestivirus RNA (Fletcher et al., 2002; Myers et al., 2001), we inserted the sequence coding for the murine ubiquitin gene between the 5'UTR and the EGFP gene. The expression level of the EGFP-N<sup>pro</sup> fusion protein from the cDNA-derived virus vA187-Ub-EGFP-N<sup>pro</sup> was 30 times lower when compared with N<sup>pro</sup> expression from the vA187-1 parent virus (Fig. 2A). However, PK-15 cells infected with vA187-Ub-EGFP-N<sup>pro</sup> were resistant to poly(IC)-mediated IFN- $\alpha/\beta$  induction to the same extent as vA187-1-infected cells, whereas poly(IC) transfection induced IFN- $\alpha/\beta$  in vA187- $\Delta$ N<sup>pro</sup>- and mock-infected PK-15 cells (Figs. 2B and D, dark gray bars). The two times higher IFN- $\alpha/\beta$  expression in mock- versus vA187- $\Delta$ N<sup>pro</sup>-infected cells (Fig. 2B) was statistically significant ( $P < 0.001$ ,  $n = 3$ ,  $t$  test). The vA187-Ub-EGFP-N<sup>pro</sup> and vA187-1-mediated block of IFN- $\beta$  induction was at the level of mRNA (Figs. 2C and D). In absence of poly(IC) transfection, vA187- $\Delta$ N<sup>pro</sup> induced IFN- $\alpha/\beta$  in PK-15 cells as previously observed, whereas no IFN- $\alpha/\beta$  expression was detected upon infection with vA187-Ub-EGFP-N<sup>pro</sup> and vA187-1, both when bioactivity and IFN- $\beta$  mRNA were analyzed (Figs. 2B and C, light gray bars). The difference in the level of IFN- $\beta$  mRNA upregulation observed 20 h post-treatment for vA187- $\Delta$ N<sup>pro</sup>-infected (Fig. 2C) versus poly(IC)-transfected cells (Fig. 2D) might reflect a difference in the kinetics of induction by the two stimuli. This was not further investigated. In SK-6 cells, vA187-Ub-EGFP-N<sup>pro</sup> prevented poly(IC)-mediated apoptosis as measured in the cell survival assay (data not shown). Taken together, these data show that CSFV expressing the EGFP-N<sup>pro</sup> fusion protein prevents poly(IC)-mediated apoptosis and IFN- $\alpha/\beta$  induction to the same extent as the parent vA187-1 virus. This validates the use of the stable cell lines expressing N<sup>pro</sup> fused to EGFP to investigate whether N<sup>pro</sup> is capable of conferring resistance to poly(IC)-mediated apoptosis and to dsRNA- and virus-mediated IFN- $\alpha/\beta$  induction, independently of other CSFV elements.

*Expression of EGFP-N<sup>pro</sup> protects SK-6 cells from dsRNA-mediated apoptosis*

We have shown previously that SK-6 cells infected with CSFV survived in the presence of higher concentrations of poly(IC) than SK-6 cells infected with CSFV lacking the N<sup>pro</sup> gene or mock-infected cells (Ruggli et al., 2003). Although the mechanism of pestivirus-mediated resistance

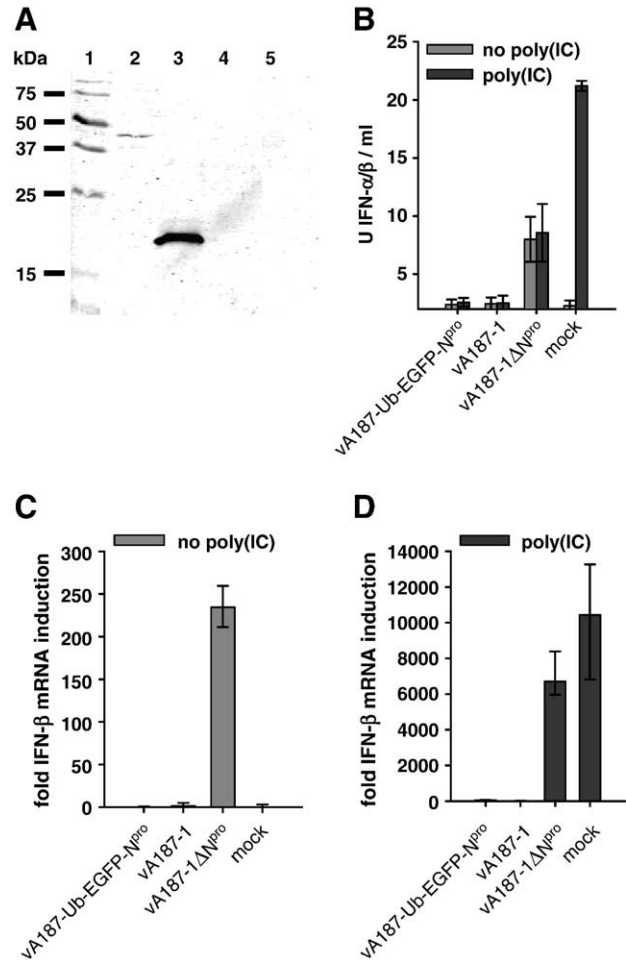


Fig. 2. CSFV vA187-Ub-EGFP-N<sup>pro</sup> maintains the IFN- $\alpha/\beta$  antagonistic phenotype of the parent vA187-1 virus. PK-15 cells were infected either with vA187-Ub-EGFP-N<sup>pro</sup>, vA187-1 or with vA187- $\Delta$ N<sup>pro</sup> or mock-treated as indicated and incubated for 65 h. (A) Total protein of infected cells was extracted with hypotonic buffer, and equal amounts of protein (5  $\mu$ g/lane) were analyzed by SDS-PAGE and Western blotting using a rabbit serum directed against N<sup>pro</sup>. Lane 1: molecular weight marker, lane 2: vA187-Ub-EGFP-N<sup>pro</sup>, lane 3: vA187-1, lane 4: vA187- $\Delta$ N<sup>pro</sup>, lane 5: mock. (B–D) Infected cells were transfected with poly(IC) or mock-transfected (no poly(IC)) as indicated. (B) Cell culture supernatant was collected 60 h post-transfection and analyzed for IFN- $\alpha/\beta$  bioactivity using the Mx/CAT reporter gene assay. The detection limit of 3 U IFN- $\alpha/\beta$  per ml is given by the lower limit of the y axis. (C–D) IFN- $\beta$  mRNA induction of infected and mock-transfected (no poly(IC)) (C) or of infected and poly(IC)-transfected PK-15 cells (D) relative to mock-treated cells was determined 20 h after transfection using quantitative TaqMan RT-PCR. For IFN- $\alpha/\beta$  bioactivity, the mean values and standard deviation from 3 independent experiments are shown (B). Error bars for mRNA induction represent minimum and maximum values of a triplicate RT-PCR (C–D).

to poly(IC) has yet to be elucidated, we analyzed whether this phenotype could be attributed to N<sup>pro</sup> alone by using constitutive expression of EGFP-N<sup>pro</sup> in SK-6 cells (Fig. 3). The cell line expressing the EGFP-N<sup>pro</sup> fusion protein had a survival rate comparable to that of CSFV vA187-1-infected cells. The percentage of surviving SK6-EGFP-N<sup>pro</sup> cells was significantly higher than the survival rate observed with mock-infected cells or with cells infected with  $\Delta$ N<sup>pro</sup> CSFV



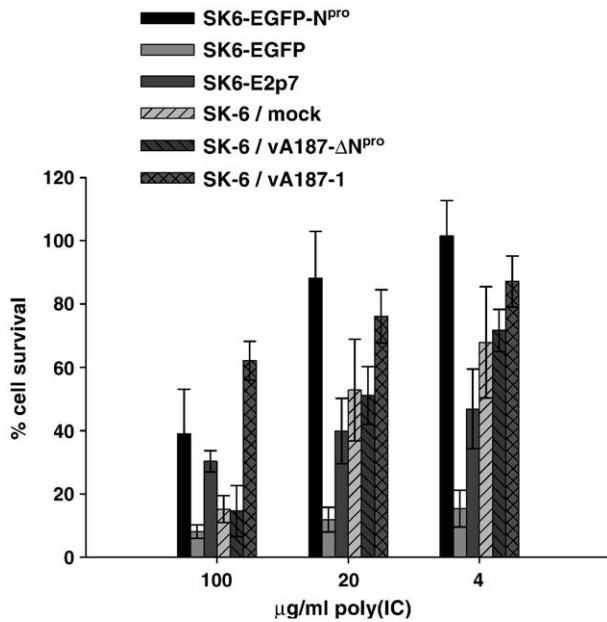


Fig. 3. Survival assay with SK-6 cells expressing EGFP-N<sup>pro</sup>, EGFP or E2p7. Stably transformed SK-6 clones constitutively expressing EGFP-N<sup>pro</sup>, EGFP or E2p7 were treated with five-fold dilutions of poly(IC) in the cell culture medium. In parallel, SK-6 cells were either mock-infected or infected with vA187-ΔN<sup>pro</sup> or vA187-1 65 h prior to poly(IC) treatment. Cell survival was monitored 72 h later by crystal violet staining. The mean values and standard deviation of 9 independent experiments are shown.

( $P < 0.01$ ,  $n = 9$ ,  $t$  test). The two cell lines expressing the proteins E2p7 of CSFV or EGFP were highly susceptible to treatment with poly(IC). For the SK6-EGFP-N<sup>pro</sup> cells, the degree of foreign protein expression was lower than for the

SK6-EGFP cells (Fig. 1) but was comparable to that of the SK6-E2p7 cells (data not shown). Thus, we can conclude that the increased survival rate to poly(IC) observed with CSFV-infected cells can be mediated by N<sup>pro</sup> independently of other CSFV elements and that this phenotype represents a significant gain of function when compared with the parent SK-6 cell line or with the SK-6 cells expressing EGFP alone.

*N<sup>pro</sup> can be complemented in trans and prevents ΔN<sup>pro</sup> CSFV- and poly(IC)-mediated IFN-α/β induction in PK-15 cells*

Recently, we associated the CSFV-mediated antagonism of IFN-α/β induction with the presence of the N<sup>pro</sup> gene. For the analysis of the effect of N<sup>pro</sup> on IFN-α/β induction, stable clones of PK-15 cells constitutively expressing EGFP-N<sup>pro</sup> fusion protein or EGFP alone as control were stimulated with ΔN<sup>pro</sup> CSFV, poly(IC) or mock. In order to exclude any artifact due to stable transformation of the cell line, two independent PK-15 clones each were tested. In the PK15-EGFP cells and the PK-15 parent cell line, ΔN<sup>pro</sup> CSFV and poly(IC) induced the secretion of IFN-α/β (Figs. 4A and B). This demonstrates that constitutive expression of a foreign protein such as EGFP does not affect the capacity of PK-15 cells to produce IFN-α/β. In the PK15-EGFP-N<sup>pro</sup> cell lines, ΔN<sup>pro</sup> CSFV did not induce any detectable IFN-α/β bioactivity, showing that the lack of N<sup>pro</sup> in ΔN<sup>pro</sup> CSFV could be complemented in *trans* (Fig. 4A). The amount of EGFP-N<sup>pro</sup> protein expressed in *trans* (Fig. 4D, lane 2) was comparable to the amount of N<sup>pro</sup> expressed in vA187-1-

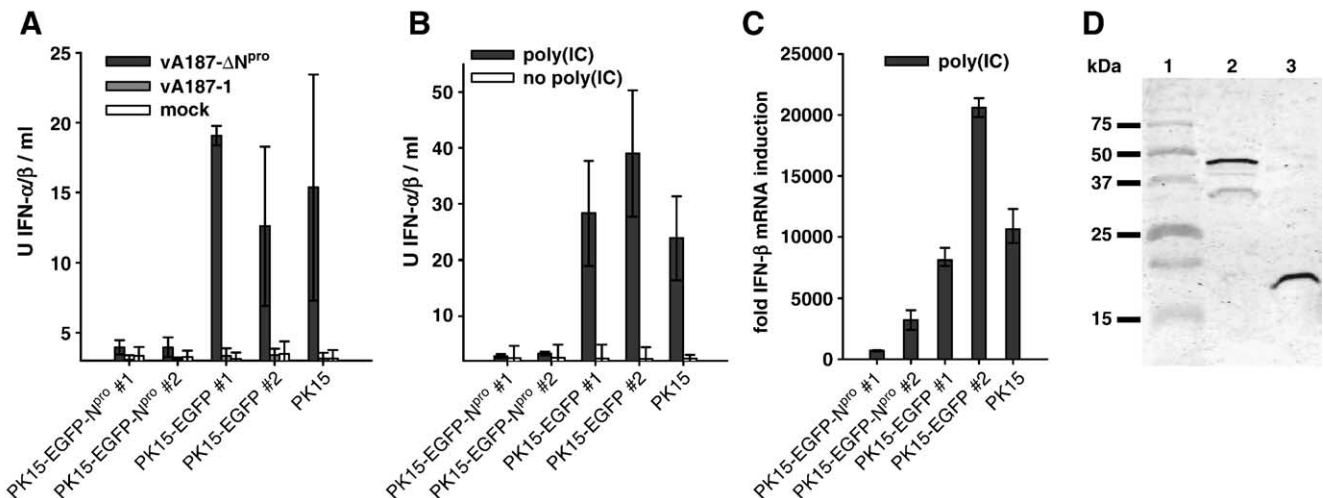


Fig. 4. Analysis of vA187-ΔN<sup>pro</sup> and poly(IC)-mediated IFN-α/β induction in PK-15 cells expressing EGFP-N<sup>pro</sup>. Two independent PK15-EGFP-N<sup>pro</sup> and PK15-EGFP clones and the PK-15 parent cells were infected at a multiplicity of infection of 2 TCID<sub>50</sub>/cell with CSFV vA187-ΔN<sup>pro</sup>, vA187-1 or mock (A) or transfected with 400 ng poly(IC)/2 × 10<sup>5</sup> cells using FuGENE 6 transfection reagent (B, C). 60 h post-infection or 40 h after poly(IC) transfection respectively, supernatants were collected and analyzed for IFN-α/β bioactivity. For IFN-β mRNA upregulation, total RNA was extracted 35 h post-transfection, and the IFN-β induction factor of poly(IC)-transfected cells relative to mock-transfected PK-15 cells was determined using TaqMan RT-PCR (C). The level of EGFP-N<sup>pro</sup> protein (D, lane 2) expressed in PK15-EGFP-N<sup>pro</sup> #1 cells was compared with the level of N<sup>pro</sup> from an equivalent amount of total protein extracted from vA187-1-infected PK-15 cells (D, lane 3). In panels A and B, the detection limit of 3U IFN-α/β/ml is given by the lower limit of the y axis. For IFN-α/β bioactivity, the mean values and standard deviation of 4 (A) and 7 (B) independent experiments are shown. Error bars for mRNA induction represent minimum and maximum values of a triplicate RT-PCR (C).

infected cells (Fig. 4D, lane 3) and completely restored the phenotype of vA187-1, resulting in absence of IFN- $\alpha/\beta$  induction. Next, we asked whether N<sup>pro</sup> was capable of blocking IFN- $\alpha/\beta$  induction independently of other CSFV elements. For this purpose, we stimulated the stably transformed cell lines with poly(IC) for IFN- $\alpha/\beta$  production. After poly(IC) transfection, no IFN- $\alpha/\beta$  bioactivity was detected in any of the N<sup>pro</sup> expressing PK-15 cell lines, demonstrating that N<sup>pro</sup> alone was capable of blocking dsRNA-mediated IFN- $\alpha/\beta$  induction in the absence of the viral context. At the level of bioactivity, no significant difference was observed between both independent clones of each type (Fig. 4B). Quantitative reverse transcription (RT)-PCR for IFN- $\beta$  mRNA after poly(IC) stimulation demonstrated that N<sup>pro</sup> was capable of inhibiting IFN- $\beta$  induction at the mRNA level (Fig. 4C). More sensitive than bioactivity detection, the quantitative RT-PCR revealed a low level of IFN- $\beta$  mRNA induction in one of the PK15-EGFP-N<sup>pro</sup> cell lines (Fig. 4C, PK15-EGFP-N<sup>pro</sup> #2). This latter experiment was performed after multiple passages of the PK15-EGFP-N<sup>pro</sup> clones. The IFN- $\beta$  mRNA upregulation in one of the two clones might reflect a loss of N<sup>pro</sup> expression in a low percentage of cells. IFN- $\alpha/\beta$  bioactivity in these cell culture supernatants was not analyzed. No porcine IFN- $\alpha$ 1 mRNA upregulation was detected in PK15-EGFP clones or in the PK-15 cells (data not shown). Accordingly, IFN- $\alpha/\beta$  bioactivity could not be neutralized from the supernatant of these cells using an anti-pig IFN- $\alpha$  serum (data not shown).

#### *N<sup>pro</sup> blocks dsRNA-mediated induction of the IFN- $\alpha$ and IFN- $\beta$ promoter in a human cell line*

The results obtained above with stably transformed PK-15 cells demonstrate that N<sup>pro</sup> prevents IFN- $\beta$  induction independently of other CSFV elements by blocking IFN- $\beta$  mRNA upregulation. Using an independent approach, we investigated the effect of transient expression of N<sup>pro</sup> and various control proteins on the poly(IC)-mediated induction of both the human IFN- $\alpha$  and IFN- $\beta$  promoter. For this purpose, we utilized established firefly luciferase reporter gene assays for human IFN- $\alpha$  and IFN- $\beta$  promoter activity in HEK293T cells, using plasmids p $\alpha$ Luc and p125Luc, respectively (Yoneyama et al., 1996). The proteins N<sup>pro</sup>, EGFP-N<sup>pro</sup>, C (Core), E<sup>ms</sup> and E2p7 of CSFV, NSs of Bunyamwera virus (BUNV) and EGFP were transiently expressed under the control of either the CMV promoter or the elongation factor 1 $\alpha$  promoter (pEAK8-derived plasmids). Protein expression was confirmed by in situ immunostaining and by Western blotting (data not shown). Two independent promoters were chosen to reduce the possibility of artifacts due to cross talk with the IFN- $\alpha$  and IFN- $\beta$  promoters. The inducible firefly luciferase expression was normalized with constitutive expression of *Renilla* luciferase using phRL-SV40 (Promega) according to Materials and methods. Similar results were obtained with p $\alpha$ Luc

(Fig. 5A) and p125Luc (Fig. 5B). In the presence of the empty expression vectors and the vectors expressing EGFP, CSFV C and E2p7 proteins, poly(IC) transfection resulted in a 3- to 5-fold induction of normalized firefly luciferase expression relative to mock transfection. Interestingly, when E<sup>ms</sup> was expressed, a slightly reduced IFN- $\alpha$  promoter induction was observed whereas the IFN- $\beta$  promoter induction was not affected. With N<sup>pro</sup> alone or in fusion with EGFP, as well as with BUNV NSs, the poly(IC)-induced IFN- $\alpha$  and IFN- $\beta$  promoter-driven firefly luciferase expression was reduced to 1-fold induction relative to mock stimulation. The inductions measured in presence of the N<sup>pro</sup> and BUNV NSs expressing plasmids were compared pairwise in the *t* test with the inductions obtained in

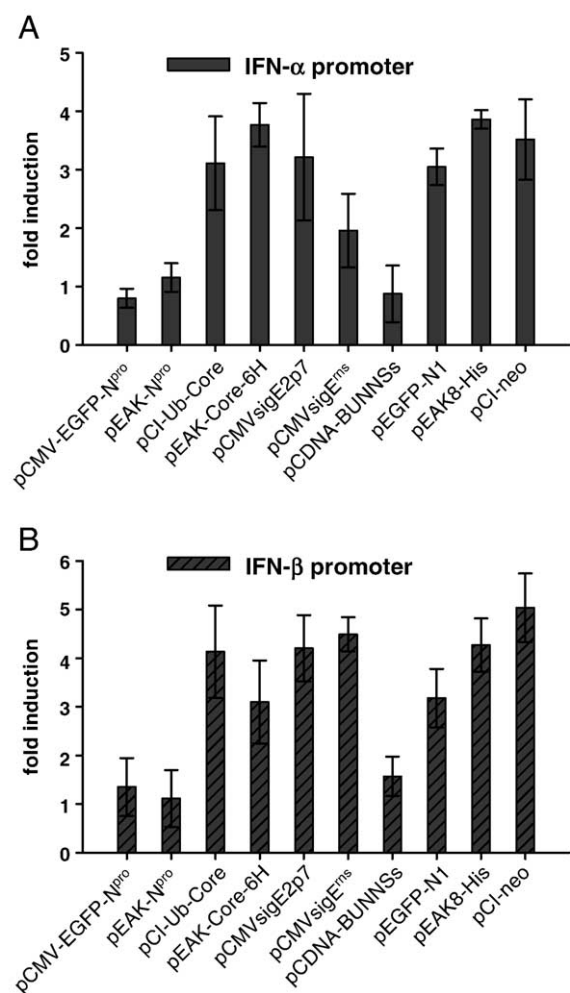


Fig. 5. Analysis of the effect of EGFP-N<sup>pro</sup> and other CSFV proteins on IFN- $\alpha$  and IFN- $\beta$  promoter induction in HEK293T cells. HEK293T cells were transfected in duplicate with a mixture of plasmid phRL-SV40 and the respective expression plasmid as indicated on the x axis and either p $\alpha$ Luc (IFN- $\alpha$  promoter) (A) or p125Luc (IFN- $\beta$  promoter) (B). After 24 h, one replica was transfected with 400 ng poly(IC) per  $2 \times 10^5$  cells, and the second replica was mock-transfected. After 18 h, the inducible firefly luciferase activity was normalized with the constitutively expressed *Renilla* luciferase activity, and the relative induction of poly(IC)-treated versus mock-treated cells was calculated. The results are the mean of 3 (A) and 6 (B) independent inductions with error bars representing the standard deviation.

presence of the plasmids expressing various other proteins or of the empty vectors. The differences were all statistically significant ( $P < 0.05$ ,  $n = 3$  for the IFN- $\alpha$  promoter and  $n = 6$  for the IFN- $\beta$  promoter). These data confirm the results obtained with the stably transformed PK-15 cells and demonstrate that N<sup>pro</sup> of CSFV prevents dsRNA-mediated upregulation of IFN- $\alpha/\beta$  mRNA transcription to the same extent as BUNV NSs. This function is specific to N<sup>pro</sup> expression since C, E<sup>ms</sup>, E2p7 and EGFP expression as well as the empty expression vectors do not significantly impair the activation of IFN- $\alpha/\beta$  promoters under the conditions analyzed.

#### EGFP-N<sup>pro</sup> prevents NDV-mediated IFN- $\beta$ induction

A well-established property of pestiviruses is the enhancement of Newcastle disease virus replication, also called END (Inaba et al., 1963; Kumagai et al., 1958). The END phenotype was related to suppression of IFN- $\alpha/\beta$  production by pestiviruses (Diderholm and Dinter, 1966; Toba and Matumoto, 1969). Therefore, we asked the question whether N<sup>pro</sup> of CSFV was responsible for the END phenotype and was capable of suppressing NDV-induced IFN- $\alpha/\beta$  production in PK-15 cells independently of other CSFV elements. For this purpose, the PK-15 clones expressing either EGFP-N<sup>pro</sup> or EGFP alone were infected with NDV at a multiplicity of infection of 0.1 50% tissue culture infectious dose (TCID<sub>50</sub>)/cell. IFN- $\beta$  mRNA upregulation was detected only in PK15-EGFP clones and in the PK-15 cell line but not in the PK15-EGFP-N<sup>pro</sup> clones (Fig. 6). IFN- $\alpha/\beta$  bioactivity in the

supernatant of NDV-infected PK-15 cells could not be measured because of the high susceptibility of the MDBK-t2 cells to NDV that could only be partially neutralized or inactivated. Nevertheless, these experiments show that N<sup>pro</sup> of CSFV can suppress IFN- $\beta$  induction mediated by an unrelated virus such as NDV.

## Discussion

The presence of the N<sup>pro</sup> gene is a feature that distinguishes the pestivirus genus from the other genera of the Flaviviridae family (for review, see Lindenbach and Rice, 2001). Autoproteolysis was the first established function of N<sup>pro</sup> (Rumenapf et al., 1998; Stark et al., 1993; Wiskerchen et al., 1991). Although N<sup>pro</sup> was found to be dispensable for pestivirus replication in cell culture (Lai et al., 2000; Tratschin et al., 1998), this gene has been maintained during evolution, and its deletion resulted in strong attenuation of the virus in pigs (Mayer et al., 2004). These observations led to the hypothesis that additional functions of N<sup>pro</sup> would confer advantage to the virus in its natural host. Recently, we obtained strong evidence that N<sup>pro</sup> was involved in the interference of CSFV with cellular antiviral defense mechanisms. The absence of N<sup>pro</sup> correlated with the loss of the capacity of the virus to interfere with dsRNA-mediated apoptosis and IFN- $\alpha/\beta$  induction (Ruggli et al., 2003). To demonstrate that N<sup>pro</sup> was by itself responsible for this interference, we established stable cell lines expressing N<sup>pro</sup> in fusion with EGFP. This approach allowed the use of control cell lines expressing the EGFP fusion partner alone to exclude effects due to expression of a foreign protein.

In order to use the EGFP-N<sup>pro</sup> fusion protein to analyze the function of N<sup>pro</sup> independently of the virus, it was necessary to demonstrate that the fusion with EGFP did not alter the function of N<sup>pro</sup> in the viral context. The experiments shown in Fig. 2 indicate that EGFP-N<sup>pro</sup> levels far below wild-type amounts of N<sup>pro</sup> are sufficient to mediate complete block of poly(IC)-mediated IFN- $\alpha/\beta$  induction. Interestingly, poly(IC) induced twice as much IFN- $\alpha/\beta$  bioactivity in mock versus  $\Delta$ N<sup>pro</sup> CSFV-infected cells (Fig. 2B). This difference might indicate the presence of additional CSFV elements that might modulate the IFN- $\alpha/\beta$  induction pathways. One candidate is the E<sup>ms</sup> protein that was shown for BVDV to specifically inactivate dsRNA-dependent signaling events when expressed in cells or added exogenously to the cell culture medium (Iqbal et al., 2004). Results from our reporter assays for IFN- $\alpha$  and IFN- $\beta$  promoter activity in HEK293T cells however suggest that E<sup>ms</sup> expression does not prevent IFN- $\alpha/\beta$  induction from an intracellular dsRNA stimulus. E<sup>ms</sup> of CSFV is found in secreted form in the supernatant of infected cells (Rumenapf et al., 1993) and possesses RNase activity (Hulst et al., 1994; Schneider et al., 1993). The secreted form of E<sup>ms</sup> expressed from  $\Delta$ N<sup>pro</sup> CSFV might account for the reduced IFN- $\alpha/\beta$  induction by poly(IC) observed in Figs. 2B and D.

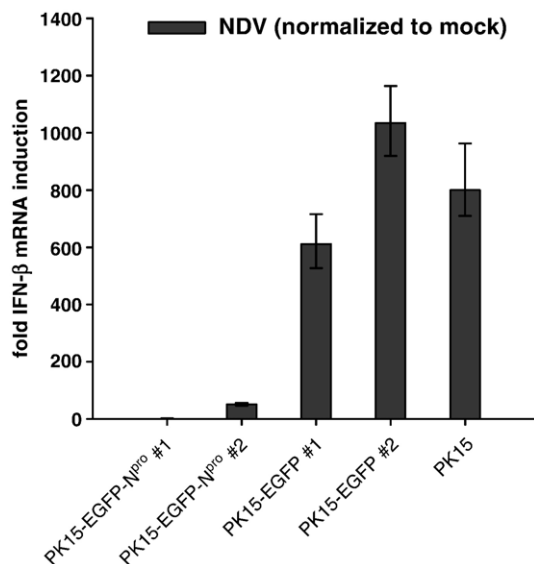


Fig. 6. Analysis of the capacity of EGFP-N<sup>pro</sup> to prevent NDV-mediated IFN- $\beta$  induction in PK-15 cells. Two independent PK15-EGFP-N<sup>pro</sup> and PK15-EGFP clones and the PK-15 parent cells were infected at a multiplicity of infection of 0.1 TCID<sub>50</sub>/cell with NDV or mock-infected. At 20 h post-infection, total RNA was extracted, and the induction factor of IFN- $\beta$  mRNA of NDV-infected cells relative to mock-infected PK-15 cells was determined using TaqMan RT-PCR. The error bars represent minimum and maximum values of a triplicate RT-PCR.



Interestingly, at the mRNA level, poly(IC) induced a much stronger response than  $\Delta N^{\text{pro}}$  CSFV (Figs. 2C and D). In PK-15 cells, the IFN- $\alpha/\beta$  bioactivity could not be blocked with anti-pig IFN- $\alpha$  serum, and no IFN- $\alpha 1$  mRNA upregulation could be detected (data not shown). This indicates that the discrepancy between IFN- $\beta$  mRNA and IFN- $\alpha/\beta$  bioactivity observed in these experiments is most probably not due to differential activation of IFN- $\beta$  and IFN- $\alpha$  but is rather due to the kinetics of induction.

Although we do not yet understand the mechanisms behind the enhanced resistance of CSFV-infected SK-6 cells to apoptosis induced by poly(IC), we demonstrate here that the expression of EGFP- $N^{\text{pro}}$  independently of the virus confers at least as good protection as infection with vA187-1. Key players in the induction of dsRNA-mediated apoptosis are the dsRNA-dependent protein kinase (PKR) and the coupled 2'–5' oligoadenylate synthetase (2–5 OAS)/RNaseL system (for reviews, see Gil and Esteban, 2000; Player and Torrence, 1998; Samuel, 2001). PKR might be directly targeted by  $N^{\text{pro}}$  or its activation may be inhibited indirectly through sequestration of dsRNA. These potential mechanisms have been described for various viral systems (for review, see Gale and Katze, 1998). Whether  $N^{\text{pro}}$  of CSFV has dsRNA binding activity is currently under investigation.

Besides being an inducer of apoptosis, it is well established that dsRNA is a potent activator of IFN- $\alpha/\beta$  production (Goodbourn et al., 2000; Jacobs and Langland, 1996). With cytopathogenic CSFV, we found a strong correlation between the amount of CSFV dsRNA and the IFN- $\alpha/\beta$  induction capacity of the virus and demonstrated that  $N^{\text{pro}}$  modulated this induction (Bauhofer et al., unpublished). Transfection experiments indicated that IFN- $\alpha/\beta$  induction was specific for in vitro synthesized CSFV dsRNA but not for non-replicating CSFV ssRNA (Bauhofer et al., unpublished). The cellular receptors for CSFV dsRNA are unknown. Potential candidates are the toll-like receptors (TLR) (for selected reviews, see Boehme and Compton, 2004; Finberg and KurtJones, 2004; Rassa and Ross, 2003) and the helicases retinoic acid inducible gene I (RIG-I) product (Yoneyama et al., 2004) and melanoma differentiation-associated gene 5 (mda-5) product (Andrejeva et al., 2004; Kang et al., 2002). All these pathways induce phosphorylation of IRF3. For noncytopathogenic and cytopathogenic BVDV, it was shown that IRF3 but not IRF7 was phosphorylated and translocated to the nucleus in response to virus infection, but IRF3 binding to cellular DNA was not observed (Baigent et al., 2002, 2004). For CSFV, La Rocca and coworkers suggested that CSFV did not provide an effective signal for nuclear translocation of IRF3 and that  $N^{\text{pro}}$  inhibited expression of IRF3 at the transcriptional level (La Rocca et al., 2005). These two reports indicate that  $N^{\text{pro}}$  of BVDV and CSFV may interfere at different levels in the IFN- $\alpha/\beta$  induction pathway. Furthermore, a recent report showed that  $N^{\text{pro}}$  of BVDV partly interfered with IRF3-dependent pathways (Horscroft

et al., 2005). We used NSs of Bunyamwera virus as control protein for the inhibition of IFN- $\alpha$  and IFN- $\beta$  induction in the transient reporter assays. This protein was shown to act downstream of IRF3 activation by inhibiting the phosphorylation of the large subunit of cellular RNA polymerase II (Thomas et al., 2004).

The interferences observed with CSFV in the viral context, i.e. resistance to poly(IC)-mediated apoptosis and prevention of IFN- $\alpha/\beta$  induction after stimulation with either poly(IC) or NDV, could be reproduced with  $N^{\text{pro}}$  alone. Thus,  $N^{\text{pro}}$  certainly represents the molecular basis for the enhancement of NDV replication by pestiviruses called END phenotype and recognized a long time ago to be due to the virus-mediated prevention of IFN- $\alpha/\beta$  induction (Diderholm and Dinter, 1966; Toba and Matumoto, 1969). The observation that  $N^{\text{pro}}$  can act independently of CSFV does not exclude the possibility that additional CSFV elements might exert a similar function and act synergistically in the same or other cell types of the host. As mentioned above, a role of  $E^{\text{ms}}$  in this context has been suggested for BVDV (Iqbal et al., 2004) and needs further investigation. The block of IFN- $\alpha/\beta$  synthesis is one of various strategies evolved by viruses to fight the interferon system (for reviews, see Goodbourn et al., 2000; Weber et al., 2004). It is increasingly clear that this strategy has also evolved in pestiviruses (Baigent et al., 2002; Bensaude et al., 2004; Carrasco et al., 2004; Ruggli et al., 2003; Schweizer and Peterhans, 2001), and  $N^{\text{pro}}$  is a key player in this context (Horscroft et al., 2005; La Rocca et al., 2005; Ruggli et al., 2003). Whether this novel function of  $N^{\text{pro}}$  depends on the proteinase activity or on other structural elements of  $N^{\text{pro}}$  or both is under current investigation. The fact that  $N^{\text{pro}}$  is also a functional antagonist of IFN- $\alpha/\beta$  mRNA upregulation in human cells opens a wide range of possibilities to analyze the pathways involved. There is no evidence yet for major interference of pestiviruses with IFN- $\alpha/\beta$  signaling and effector pathways (Peterhans et al., 2003; Schweizer and Peterhans, 2001). For BVDV, it was shown that the IFN-stimulated MxA biosynthesis was not compromised in noncytopathogenic BVDV-infected cells (Baigent et al., 2002). This is in contrast with other Flaviviridae (Diamond, 2003) and with the closely related HCV that interfere with IFN signaling (for review, see Macdonald and Harris, 2004; Tan and Katze, 2001). More detailed understanding of the mechanisms of how  $N^{\text{pro}}$  interferes with the IFN- $\alpha/\beta$  induction pathways and affects virus replication in vivo is expected to shed more light on the pathogenesis of classical swine fever.

## Materials and methods

### Cells and viruses

The porcine kidney cell line SK-6 (Kasza et al., 1972) was obtained from M. Pensaert (Faculty of Veterinary Medicine, Ghent, Belgium) and was propagated in Earle's minimum

essential medium (EMEM) supplemented with 7% horse serum. The porcine kidney cell line PK-15 (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's minimum essential medium supplemented with 5% horse serum. HEK293T cells were propagated in EMEM supplemented with 7% fetal bovine serum (Biocrom AG, Switzerland). The MDBK-t2 cell line was kindly provided by Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, UK) and maintained in EMEM supplemented with 7% fetal bovine serum and 10 µg/ml blasticidin (Invitrogen). CSFV strains vA187-1 and vA187-ΔN<sup>pro</sup> were derived from the full-length cDNA clones pA187-1 (Ruggli et al., 1996) and pA187-ΔN<sup>pro</sup> (Ruggli et al., 2003), respectively. CSFV vA187-Ub-EGFP-N<sup>pro</sup> was rescued from pA187-Ub-EGFP-N<sup>pro</sup>, a full-length cDNA clone in which a cassette encoding the murine ubiquitin, the EGFP and a Gly–Gly–Ser–Gly–Gly–Ser peptide linker, was inserted between the 5'UTR and the N<sup>pro</sup> gene of pA187-1 (details of the construction can be obtained on request). All cDNA-derived viruses were rescued by electroporation of SK-6 cells with the respective in vitro transcripts as described elsewhere (Moser et al., 1999). NDV strain Hitchner B1 was obtained from Lohmann AG (Cuxhaven, Germany) and passaged once on specific pathogen-free eggs resulting in a titer of 10<sup>6.5</sup> TCID<sub>50</sub>/ml on PK-15 cells.

### Plasmids

The reporter plasmids pαLuc and p125Luc expressing firefly luciferase under the control of human IFN-α and IFN-β promoter elements respectively were a gift of Takashi Fujita (Yoneyama et al., 1996). Plasmid pRL-SV40 (Promega) constitutively expressing *Renilla* luciferase was used for internal normalization of inducible firefly luciferase. The three expression plasmids pEGFP-N1 (Clontech), pEAK8-His derived from pEAK8 (Edge BioSystems) as described elsewhere (Balmelli et al., 2005) and pCI-Neo (Promega) were used to express the proteins N<sup>pro</sup>, C and E2p7 of CSFV Alfort/187. The respective CSFV genes were derived from the full-length cDNA clone pA187-1 (Ruggli et al., 1996) and engineered into the expression plasmids by standard cloning technique using PCR with PfuTurbo DNA polymerase (Stratagene). For the construction of pCMV-EGFP-N<sup>pro</sup>, the N<sup>pro</sup> gene was amplified with the sense primer Bsr-GS-NproL (5'-GAGCTGTACAAGGGCG-GAAGCGGAGGCAGCATGGAGTTGAATCATTTT-GAACT-3', containing the 3' end of the EGFP gene including the restriction site *Bsr*GI but lacking the stop codon followed by a sequence encoding a Gly–Gly–Ser–Gly–Gly–Ser peptide linker and by the 5'-terminal sequence of the N<sup>pro</sup> gene) and with the antisense primer N-st-NproR (5'-ATATAGCTGCGGCCGCTAATTAAT-CAGCAACTGGTAACCCACAATGGA-3', encoding the antisense sequence of the 3' end of the N<sup>pro</sup> gene, three stop codons and a *Not*I restriction site). PCR-based cloning was also used for the construction of pEAK-N<sup>pro</sup>, pEAK-

Core-6H, pCI-Ub-Core, and pCMVsigE<sup>ms</sup> (details can be obtained on request). The construct pCMVsigE2p7 was described elsewhere (Maurer et al., 2005). Plasmid pCDNA-BUNNSs (Weber et al., 2002) kindly provided by Friedemann Weber was used to express NSs of BUNV. Plasmids were propagated in *E. coli* XL-1 blue (Stratagene) and purified using the NucleoBond Plasmid DNA purification system (Macherey-Nagel). All constructs were verified by DNA sequencing using the Thermo Sequenase DYEnamic direct cycle sequencing kit (Amersham Biosciences) and the Global IR<sup>2</sup> System and e-Seq software (LI-COR).

### Selection of stably transformed cell lines

Subconfluent monolayers of SK-6 and PK-15 cells were transfected with pCMV-EGFP-N<sup>pro</sup> or with pEGFP-N1 using FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol with a 6 to 1 (v/w) FuGENE 6 to DNA ratio. The plasmid DNA was added to serum-free EMEM containing the FuGENE 6 transfection reagent. After 20 min of incubation at room temperature, the transfection mixture was added directly into the complete culture medium of the cell monolayer. After 48 h, the cell culture medium was replaced with fresh complete medium supplemented with 500 µg/ml G418-Sulfate (Calbiochem-Novabiochem Corp). Single colonies were picked and purified by two to three rounds of end point dilution. The purified clones were propagated under selection with 250 µg/ml G418-sulfate.

### Antibodies

For the detection of the viral protein N<sup>pro</sup>, a polyclonal antiserum was produced in rabbits using recombinant N<sup>pro</sup> expressed in *E. coli*. For this purpose, the N<sup>pro</sup> gene of CSFV strain vA187-1 was amplified from pA187-1 using the oligonucleotide B-NproL (5'-GCGAGCGGATCC-GATGGAGTTGAATCATTTTGAAT-3') containing a *Bam*HI site upstream of the start codon and the antisense oligonucleotide H-st-NproR (5'-CTAGCTAAGCTTAG-CAACTGGTAACCCACAATGGA-3') coding for a stop codon and a *Hind*III recognition site downstream of the N<sup>pro</sup> sequence. The PCR fragment was cloned into pCR-XL-TOPO (Invitrogen), and the DNA sequence was verified by nucleotide sequencing as described above. The N<sup>pro</sup> gene was then excised with *Bam*HI and *Hind*III restriction endonuclease digestion and subcloned into the *Bam*HI and *Hind*III restriction sites of the bacterial protein expression plasmid pQE-31 (QIAGEN), in frame with a 5'-terminal sequence encoding six histidine residues. The resulting plasmid pQE-6H-N<sup>pro</sup>A187 was then used to transform *E. coli* M15 cells, and the recombinant N<sup>pro</sup> protein was expressed by induction of a log phase culture with isopropyl β-D-thiogalactopyranoside (IPTG) for 5 h at 37 °C. For protein purification by nickel chelate affinity chromatography, cells were lysed in denaturing lysis buffer (8 M Urea,



100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris–HCl, pH 9.6) and purified using an AEKTA FPLC apparatus (Amersham Biosciences) and prepacked 1-ml HiTrap chelating columns (Amersham Biosciences), according to the manufacturer's protocol. For antiserum production, New Zealand white rabbits (Charles River Laboratories) were immunized three times at 3-week intervals with 0.5 mg purified N<sup>pro</sup> mixed 1:1 with complete Freund's adjuvant (Sigma) for the first immunization and incomplete Freund's adjuvant (Sigma) for the two booster injections. The mAb JL-8 (BD Biosciences Clontech) was used for the detection of EGFP by Western blotting. Alexa Fluor 680 goat anti-mouse and anti-rabbit IgG were obtained from Molecular Probes Inc.

#### *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting*

Cellular proteins were extracted with a hypotonic buffer (20 mM morpholinepropanesulfonic acid, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1% Triton X-100, pH 6.5). Total protein was quantified using a bicinchoninic acid-based assay (Pierce), and the concentration was adjusted to 1 mg/ml. Proteins were separated by SDS-PAGE under reducing conditions according to standard protocols (Sambrook et al., 1989). After SDS-PAGE, gels were equilibrated for 30 min in SDS transfer buffer (12.5 mM Tris–HCl, 125 mM glycine, 0.05% SDS, 20% methanol, pH 8.3), and the proteins were transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad) in SDS transfer buffer at 15 V for 30 min using a Trans-Blot SemiDry transfer device (BioRad). The membranes were then washed briefly in PBS (80 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, pH 7.3) and blocked for 1 h at room temperature or overnight at 4 °C with Odyssey Blocking Reagent (LI-COR) diluted 1:1 with PBS. For immunodetection, the membranes were incubated for 30 min at room temperature with primary and secondary antibodies diluted in Odyssey Blocking Reagent (LI-COR) mixed 1:1 with PBS. After each incubation period, the membranes were washed with PBS supplemented with 0.1% Tween 20. Two final wash steps with PBS lacking detergent were carried out prior to image acquisition and protein quantification using the Odyssey Infrared Imaging System (LI-COR).

#### *Transfection of poly(IC)*

For IFN- $\alpha/\beta$  induction, cells were transfected with poly(IC) (Sigma) using FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. For each  $5 \times 10^5$  cells to be transfected, 6  $\mu$ l FuGENE 6 reagent was mixed in final volume of 100  $\mu$ l serum-free EMEM prior to addition of 1  $\mu$ g poly(IC), resulting in a 6 to 1 (v/w) ratio of FuGENE 6 reagent to nucleic acid. After 20 min of incubation at room temperature, the transfection mixture was added to the cells directly into the complete culture medium.

#### *Interferon assay*

Type I IFN activity was assayed as previously described (Ruggli et al., 2003) using an Mx/CAT reporter gene assay developed for the quantification of bovine IFN- $\alpha/\beta$  and kindly provided by Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, UK) (Fray et al., 2001). This assay was validated for the detection of porcine IFN- $\alpha/\beta$  bioactivity as described elsewhere (Ruggli et al., 2003). Briefly, MDBK-t2 cells maintained under blasticidin selection were seeded in 6-well plates at a density of  $10^6$  cells/well. After 24 h, samples diluted 1:5 or recombinant porcine IFN- $\alpha$  standard produced in HEK293-EBNA cells (Balmelli et al., 2005) was added to the cells in 1 ml EMEM supplemented with 7% heat-inactivated FCS. The cells were then incubated for 24 h prior to lysis and quantification of chloramphenicol acetyltransferase (CAT) by CAT ELISA (Roche). Infectious CSFV present in the samples was neutralized by incubation for 1 h at 4 °C with polyclonal pig anti-CSFV serum 600-88 (Ruggli et al., 1995) diluted 1:100. Virus inactivation was monitored on SK-6 cells using immunohistochemistry with mAb HC/TC26 (Greiser-Wilke et al., 1990). Poly(IC) was removed from the samples by incubation at 37 °C for 1 h with a cocktail of 5 U/ml RNase A and 200 U/ml RNase T1 (Ambion). For neutralization of IFN- $\alpha$ , 250  $\mu$ l of sample was incubated for 30 min on ice with 1  $\mu$ l rabbit polyclonal antibody against porcine IFN- $\alpha$  (>250 neutralization U/ $\mu$ l as determined by the manufacturer, PBL Biomedical Laboratories).

#### *Cellular RNA isolation*

For IFN- $\beta$  mRNA quantification, total RNA was extracted from cells using the NucleoSpin RNA II extraction kit (Macherey-Nagel). Typically, monolayers of  $10^6$  cells were lysed according to the manufacturer's protocol, including the DNase digestion step. The purified RNA was eluted with 60  $\mu$ l RNase-free water.

#### *Quantitative RT-PCR for porcine IFN- $\beta$ mRNA*

Oligonucleotides for the quantification of porcine IFN- $\beta$  mRNA by TaqMan real time RT-PCR were designed based on the published porcine IFN- $\beta$  coding sequence (GenBank accession nos. S41178 and M86762) (Artursson et al., 1992) using the Primer Express version 1.5 software (Applied Biosystems). The IFN- $\beta$  mRNA was amplified with sense primer IFNbeta-L (5'-GGCTGGAATGAAACCGTCAT-3') and antisense primer IFNbeta-R (5'-TCCAGGATTGTCTC-CAGGTCA-3') in combination with the 5'FAM/3'TAMRA-labeled TaqMan probe IFNbeta-P (5'-CCTTGTGGAACCTT-GATGGGCAGATGG-3'). For internal normalization with GAPDH mRNA, the sense primer porcGAPDH-F (5'-CATCACTGCCACCCAGAAGA-3') and antisense primer porcGAPDH-R (5'-ATGATGTTCTGGGCAGCCC-3') were used together with the 5'TET/3'TAMRA-labeled porc-

GAPDH-P (5'-TGTGGATGGCCCGTCTGGGAA-3') designed from the porcine GAPDH nucleotide sequences with GenBank accession nos. AF141959, AF202970 and U82261. Real-time RT-PCR was carried out using the TaqMan Gold RT-PCR Kit (PE Applied Biosystems). The two-step RT-PCR consisted of a reverse transcription and of a parallel control reaction in which the reverse transcriptase was omitted (no RT) followed by triplicate real-time TaqMan PCR. The 20 µl RT mixes contained RNA from approximately 10,000 cells in 1× TaqMan Buffer A, 5.5 mM MgCl<sub>2</sub>, 500 µM of each dNTP, 2.5 µM random hexamers, 0.8 U/µl RNasin (Promega) and 1.25 U/µl MultiScribe reverse transcriptase and were kept for 10 min at room temperature followed by incubation at 48 °C for 30 min and enzyme inactivation at 95 °C for 10 min. Then, 4 µl of the RT reaction or of the no RT control reaction was added to the PCR mix consisting of 1× TaqMan Buffer A, 4.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 400 nM of the respective sense and antisense primer, 100 nM of the corresponding dual-labeled TaqMan probe and 0.02 U/µl AmpliTaq Gold DNA polymerase in a final volume of 25 µl. The real-time PCR was performed in a ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following cycling profile: 10 min at 95 °C (activation of the AmpliTaq Gold DNA polymerase) followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C. The IFN-β mRNA induction was calculated by subtracting the mean C<sub>T</sub> value obtained for the RT-PCR from the C<sub>T</sub> value of the no RT control PCR (residual DNA). The individual samples were normalized to each other using the respective C<sub>T</sub> value for GAPDH mRNA.

#### *Assay for cell survival after poly(IC) treatment*

Survival of SK-6 cells to poly(IC) treatment was monitored as previously described (Ruggli et al., 2003). Briefly, CSFV- or mock-infected SK-6 cells or stably transformed SK-6 cell clones were treated either with serial dilutions of poly(IC) (Sigma) or mock-treated. Cell survival was monitored 72 h after poly(IC) treatment by crystal violet staining. The percentage of optical density of poly(IC)-treated versus mock-treated cultures was expressed as percent cell survival.

#### *Reporter gene assays*

For the reporter gene assays, 2 × 10<sup>5</sup> HEK293T cells per well of a 24-well plate were transfected with a mixture of 400 ng of inducible reporter plasmid pαLuc or p125Luc, 2 ng of plasmid pRL-SV40 for internal normalization and 5 ng of the respective expression plasmid. DNA transfections were performed with FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol as described above using a 6 to 1 (v/w) FuGENE 6 to DNA ratio. Cells were incubated for 24 h at 37 °C and then transfected with 400 ng poly(IC) per well or mock-

transfected as described above and incubated for additional 18 h prior to extraction and quantification of firefly and *Renilla* luciferase using the Dual Luciferase Reporter assay system (Promega) and a Lumat LB 9507 Luminometer with two automated injectors (Berthold Technologies).

#### *Statistical analysis*

Statistical and graphical analysis was performed using the SigmaStat and SigmaPlot software package (SPSS).

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